

β -Crystallins insolubilized by calpain II in vitro contain cleavage sites similar to β -crystallins insolubilized during cataract

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Incubation of soluble proteins from rat lens with the protease calpain II caused the precipitation of β -crystallin polypeptides. Two-dimensional electrophoresis and sequence analysis identified β -crystallin polypeptides both before and after their precipitation by calpain II. β -crystallin polypeptides precipitated by calpain were cleaved at their NH₂-terminal extensions. These cleavage sites were similar to cleavage sites occurring in β -crystallin polypeptides precipitated during formation of experimental cataract induced by an overdose of selenite. These data suggested that calpain II caused β -crystallin insolubilization during cataract formation, and indicated that the process can be mimicked in vitro.

Crystallin; Cataract; Calpain

1. INTRODUCTION

The majority of the dry weight of the lens of the eye is composed of proteins called crystallins. In the mammalian lens, these proteins are divided into three major classes: α -, β -, and γ -crystallins [1]. These divisions are based on the elution of soluble lens proteins during gel filtration. Native α -crystallin is an oligomer of approximately 800 kDa, and is composed of primarily two 20 kDa subunits named α A and α B. Native β -crystallins are also oligomers, which elute in two or more size classes during gel filtration, and range from 200–50 kDa. They contain 7 different types of subunits, named β B1, β B2, β B3, β A1, β A2, β A3, and β A4, ranging from 31–23 kDa. To date, genes coding for all 7 of the major β -crystallin subunits have been sequenced [2–5]. β -Crystallin polypeptides share sequence and structural homologies with γ -crystallins, reflecting their common evolutionary origin [1], however, unlike β -crystallins, γ -crystallins do not form oligomers, and contain no extensions at either their NH₂- or COOH-termini [1].

Crystallins have the remarkable ability of existing in high concentrations in the transparent lens without causing light scatter [6], however, this property is lost if lens crystallins become denatured. Crystallin denaturation is most readily observed during formation of experimental cataracts by the loss of their solubility in water [7].

Recent data in rodents suggests that removal of por-

tions of the NH₂-terminal extensions of β -crystallins by the calcium-dependent neutral protease calpain II (EC 34.22.17) may contribute to crystallin denaturation and insolubilization during cataract. This hypothesis is supported by three observations.

(i) When 10-day-old rats are injected with a single overdose of selenite, their lenses undergo an elevation in calcium and form a dense opacity within 4 days [8]. The opaque insoluble protein from these lenses contained an abundance of partially degraded β -crystallin polypeptides missing from 5–49 amino acids from their NH₂-terminal extensions [9]. Calpain II was probably responsible for this partial proteolysis, since intact β -crystallin polypeptides were cleaved by purified calpain II at sites identical to cleavage sites appearing during cataract formation [9].

(ii) Cataracts produced in cultured rat lenses by addition of the cataractogenic agents selenite, calcium ionophore A23187, xylose, and diamide all underwent partial degradation of β -crystallin polypeptides and opacification. Addition of E64 or E64d, inhibitors of calpain and other cysteine proteases, decreased proteolysis induced by the various toxins, and temporally prevented the subsequent opacification [10,11].

(iii) Incubation of soluble lens crystallins with purified calpain II in vitro caused a rapid increase in turbidity, due to the insolubilization of partially degraded β -crystallin polypeptides. When examined by two-dimensional electrophoresis, the polypeptides insolubilized by calpain II migrated to positions that were identical to positions of partially degraded β -crystallin polypeptides appearing in the insoluble protein of cataractous lenses [12].

The finding that calpain II caused the insolubilization

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of crystallins in vitro was important, because it provided a model to study the mechanism of crystallin insolubilization during cataract. The purpose of the present study was to examine the validity of the in vitro insolubilization model by determining cleavage sites on β -crystallins following calpain-induced insolubilization. We found that β -crystallins insolubilized by calpain in vitro were cleaved at the same sites on their NH_2 -terminal extensions as β -crystallins insolubilized during cataract. These data confirmed that in vitro insolubilization by calpain II is a useful model to study the mechanism of crystallin insolubilization during cataract formation.

2. MATERIALS AND METHODS

The lenses of 2-week-old Sprague-Dawley rats were dissected and homogenized at a ratio of 1 mg lens wet weight/2.5 μl buffer A containing 20 mM imidazole (pH 6.8), 0.1 mM EGTA, and 2.0 mM dithioerythritol (DTE). The soluble protein was isolated by centrifugation of the lens homogenates at $10,000 \times g$ for 15 min at 25°C . Calpain II was purified from porcine heart muscle and assayed as previously described [12]. Soluble lens protein at 30 mg/ml concentration was incubated for 60 min in buffer A containing 5 U calpain II/mg lens protein and 1.0 mM CaCl_2 . The increase in turbidity during crystallin insolubilization was measured in the wells of a microtiter plate, and the extent of insolubilization was similar to previous experiments [12]. The resulting precipitated proteins were isolated by centrifugation at $10,000 \times g$ for 10 min at 25°C , washed once in buffer A containing no DTE, resuspended in water, a portion removed for protein assay using bovine serum albumin as a standard (bicinchoninic acid method; Pierce Chemical Company), and the remainder dried by vacuum centrifugation. Insoluble protein from the opaque lens nucleus of rats with selenite-induced cataracts was isolated as before [9].

Two-dimensional electrophoresis of proteins was carried out as before [12], followed by blotting onto polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore, Bedford, MA) [9]. Blots were stained for 1 min in 0.1% Ponceau S, 1% acetic acid, and rinsed

with MilliQ water (Millipore) to remove background stain. Individual polypeptides were then cut out, destained in pH 11 NaOH solution, rinsed with MilliQ water, then stored at -20°C . Six NH_2 -terminal amino acid residues were then determined from combined spots from 2–4 gels (100 μg total protein/gel) using an Applied Biosystems model 470A protein-sequencer, and model 120 PTH analyzer.

Polypeptides with blocked NH_2 -termini were identified by sequencing tryptic fragments produced by in situ digestion of blotted crystallin polypeptides on PVDF membranes. The polypeptides were separated by two-dimensional electrophoresis as above, except 125–250 μg total protein was applied, and spots from 3–16 gels were pooled. Membranes were then blocked by incubation for 30 min in 0.5% polyvinylpyrrolidone-40, 1% acetic acid, followed by ten MilliQ water rinses. The blotted polypeptides were then incubated for 24 h at 37°C in 50 μl 0.1 M NH_4HCO_3 (pH 8.0), 10% acetonitrile, and 4 μl of 1 mg/ml sequencing grade modified trypsin (Promega Corp., Madison, WI) dissolved in 0.1% trifluoroacetic acid (TFA). The tryptic fragments released from the membranes were then dried by vacuum centrifugation, dissolved in 0.1% TFA, and injected onto a 150×2.1 mm C_{18}

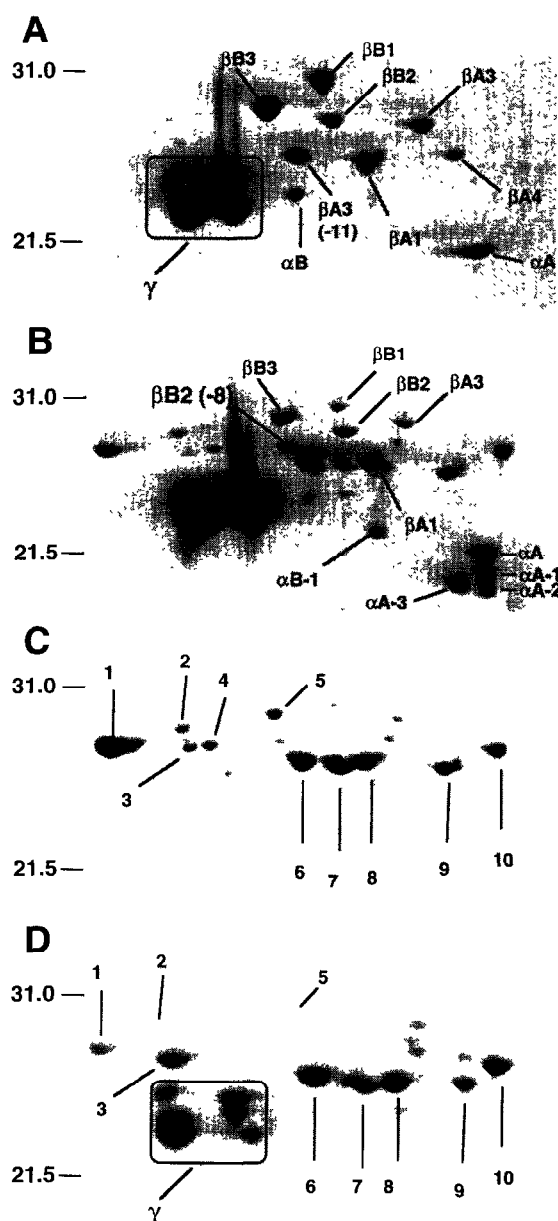


Fig. 1. Two-dimensional electrophoresis of lens crystallins before and after partial degradation by calpain. (A) Soluble crystallin polypeptides from the lens of 2-week-old control rats. All α - and β -crystallin fragments appeared blocked, with the exception of $\beta\text{A}3$ (-11), which, based on its NH_2 -terminal sequence, was $\beta\text{A}3$ missing 11 amino acids from its NH_2 -terminal extension. The identification of all other major α - and β -crystallin polypeptides was based on the sequence of fragments produced by digestion of electroblotted polypeptides with trypsin (the tryptic maps for the β -crystallins are shown in Fig. 2). (B) Crystallin polypeptides from the lens of 2-week-old rats which remained soluble following incubation with calpain II. The positions of the remaining undegraded β -crystallin subunits are indicated, as well as the identity of partially degraded α - and β -crystallin polypeptides which, unlike the other proteolytic fragments which appeared, were not partially insolubilized during calpain incubation. (C) β -Crystallin polypeptides undergoing precipitation during incubation with calpain. The major insolubilized fragments were numbered and identified by Edman sequencing of their NH_2 -terminus, and/or analysis of tryptic fragments (Table I, Fig. 3). (D) Insoluble protein from the opaque nuclear region of rat lens 4 days following selenite injection. Polypeptides with positions similar to polypeptides found in C were given the same numbers so that their identities could be compared (Table I). The boxed region shows γ -crystallin polypeptides were appeared in the insoluble fraction prior to cataract formation [9].

column (Vydac, Hesperia, CA). Chromatography was carried out at 50°C, using a 0.2 ml/min flow rate, and linear 75 min 0–55% acetonitrile gradient containing 0.1% TFA. Tryptic fragments were detected by absorbance at 216 nm, and 1 min fractions collected. Tryptic fragments were then identified by determining the sequence of 6–10 NH₂-terminal amino acid residues as above.

3. RESULTS

Soluble lens crystallins from normal 2-week-old rat lens were separated by two-dimensional electrophoresis, and 7 major β -crystallin polypeptides, and 2 major α -crystallin polypeptides were identified (Fig. 1A). The

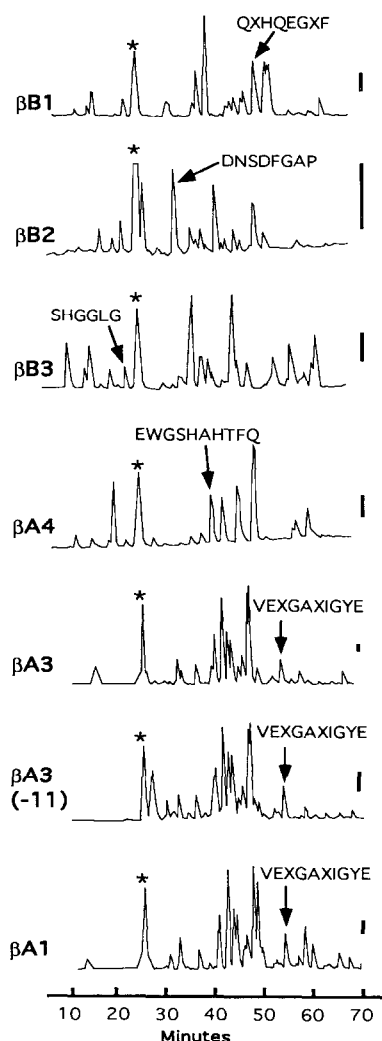


Fig. 2. HPLC separation of tryptic fragments of control β -crystallin polypeptides shown in Fig. 1A. Each polypeptide was identified by 6–10 cycles of Edman sequencing of the tryptic fragments indicated by arrows. The polypeptides were then identified by comparing the given sequence to published sequences of β -crystallins [2–5]. Due to their similar sequence, β A3 and β A1 were differentiated by their relative molecular weights (Fig. 1A), and β A3 (–11) was identified by its NH₂-terminal sequence. The asterisk indicates the elution position of trypsin. The bar on the right indicates 0.01 absorbance units at 216 nm. X indicates the position of cysteine or tryptophane residues which were not determined.

γ -crystallin polypeptides (Fig. 1A, boxed region) were not analyzed, because they were not calpain substrates. The α - and β -crystallin polypeptides from these normal lenses could not be identified by simple NH₂-terminal sequence analysis, because they were NH₂-terminally blocked. An exception was β A3 (–11). The sequence of the six NH₂-terminal amino acids of polypeptide β A3 (–11) were TLPTTK, confirming its identity as β A3 crystallin missing 11 amino acids from its NH₂-terminus [4,9]. The remaining β -polypeptides were identified by trypsinization, separation of fragments by HPLC, and determination of the sequence of 8–10 amino acids from individual fragments (Fig. 2). The tryptic map of each polypeptide was distinct, with the exception of β A3, β A3 (–11), and β A1, which had similar tryptic maps. The similarity of the tryptic maps and sequence of tryptic fragments confirmed that β A3, β A3 (–11), and β A1 were all products of the single β A3/A1 gene [4]. The lowest molecular weight of the three, β A1, is synthesized using an alternate initiation codon, resulting in a polypeptide 17 amino acids shorter than β A3 [4], while β A3 (–11) was probably produced by post-translational processing of β A3. The identities of α A and α B were similarly determined by fragmentation and sequencing of tryptic fragments (data not shown). Therefore, all α - and β -crystallin polypeptides were identified, with the exception of β A2 and α A (insert) [1], which were not major components in 2-week-old rat lens.

Soluble lens crystallins from 2-week-old rat lens were then incubated with calpain II for 60 min. As previously described, the turbidity of the incubation mixture increased due to insolubilization of approximately 4% of the total protein [12]. Following centrifugation of the incubation mixture, the resulting supernatant and pellet were separated by two-dimensional electrophoresis. Many new partially degraded polypeptides appeared in both the supernatant (Fig. 1B) and pellet (Fig. 1C). Changes in the relative concentrations of polypeptides during calpain incubation suggested that the majority of new polypeptides in both the supernatant and pellet were derived from partial degradation of β B1, β B3, β A3, β A4, α A, and α B. Furthermore, examination of the proteins insolubilized by calpain suggested that they were entirely composed of polypeptides, which, because of their apparent molecular weight, were all partially degraded β -crystallin polypeptides (Fig. 1C).

This hypothesis was confirmed by direct sequence analysis of the polypeptides precipitated by calpain. Polypeptides 2, 3, 4, 6, 9, and 10 (Fig. 1C) were identified as partially degraded β B1, β B3, β A3, and β A4 missing portions of their NH₂-terminal extensions (Table I). Insoluble polypeptides 1 and 7 (Fig. 1C) could not be identified by direct sequence analysis, due to indistinct NH₂-termini. Therefore, each was identified by sequencing tryptic fragments (Fig. 3). Insoluble polypeptide 1 was identified as β B3 missing a portion of its NH₂-terminus. This was based on finding the complete

sequence of the COOH-terminal tryptic fragment of β B3 (Fig. 3, Polypeptide 1). Furthermore, the appearance of alanine in the first two cycles during direct sequencing of untrypsinized polypeptide 1 suggested the calpain cleavage site was at or near residue 10 [3]. Similarly, polypeptide 7 was identified by the amino acid sequence of an internal tryptic fragment as a partially degraded β A3 or β A1 (Fig. 3, Polypeptide 7). Direct sequence analysis of the untrypsinized polypeptide then suggested that insoluble polypeptide 7 was derived by removal of a range of 17–21 amino acids from the NH₂-terminus of β A3. Insoluble polypeptides 5 and 8 appeared blocked at their NH₂-terminus, however, their positions suggested that they were intact β B3 and β A1, respectively (compare Fig. 1C with Fig. 1A). This was subsequently confirmed by the similarity of their tryptic maps (data not shown) to the tryptic maps of intact β B3 and β A1 (Fig. 2). The insolubilization of intact β B3 and β A1 during incubation with calpain illustrated that partial degradation was not always required for insolubilization to occur. Intact β A1 may have precipitated due to interaction with other β -crystallin subunits which were partially degraded.

Comparison of polypeptides insolubilized by calpain (Fig. 1C) with those remaining soluble following calpain incubation (Fig. 1B) indicated that a significant proportion of partially degraded β -crystallin polypeptides identified in the pellet remained soluble. This comparison also indicated that some partially degraded polypeptides did not undergo significant insolubilization. Five of these partially degraded crystallins which remained soluble were identified. These included: β B2 missing 8 amino acids from its NH₂-terminus (Fig. 1B,

β B2–8), identified based on its NH₂-terminal sequence AGKPQP [5]; and polypeptides α A–1, α A–2, α A–3, and α B–1 (Fig. 1B), identified as partially degraded α A and α B polypeptides by similarity of their tryptic maps to the tryptic maps of intact α A and α B polypeptides (data not shown). NH₂-Terminal sequence analysis of these partially degraded polypeptides also indicated that they remained NH₂-terminally blocked, suggesting that they were produced by cleavage at the COOH-terminus of both α A and α B.

The polypeptides insolubilized by calpain in vitro (Fig. 1C) were then compared to polypeptides insolubilized in vivo during formation of selenite induced cataract (Fig. 1D). Many of the polypeptides insolubilized by calpain II in vitro migrated to the same positions, and contained the same NH₂-terminal cleavage sites as polypeptides insolubilized during formation of cataract (Table I), however, there were minor differences between the insoluble protein formed in vitro (Fig. 1C) and the insoluble protein formed during cataract (Fig. 1D). The insolubilization during cataract was accompanied by more extensive degradation of β B3 than during insolubilization in vitro. For example, polypeptide 1 (β B3 missing approximately 10 residues) was the most abundant β B3 fragment in the protein insolubilized by calpain in vitro, while polypeptide 3 (β B3–17) was the most abundant β B3 fragment in protein insolubilized during cataract formation. Another example of more extensive degradation during cataract formation was illustrated by the identities of polypeptides numbered 8 in both Fig. 1C and D. Polypeptide 8 insolubilized by calpain in vitro remained NH₂-terminally blocked, and was identified as intact β A1, while polypeptide 8 insolubilized during cataract was derived by removal of either 4 or 21 amino acids from β A1 or β A3, respectively, and deamination of the resulting NH₂-terminal asparagine [9].

4. DISCUSSION

The major finding in the present study was that partially degraded β -crystallin polypeptides insolubilized by calpain II in vitro were cleaved at similar sites at their NH₂-terminal extensions as β -crystallin polypeptides insolubilized during cataract formation in vivo. This extends our earlier calpain cleavage site data for β -crystallins [9], and further supports the hypothesis that removal of NH₂-terminal extensions of β -crystallins by calpain is directly responsible for β -crystallin insolubilization during cataract formation. The data also suggested that in vitro insolubilization by calpain is a useful model to study crystallin insolubilization during experimental cataract.

The present study is also the first to demonstrate the utility of in situ tryptic digestion to identify crystallins following their separation by two-dimensional electrophoresis. The methodology was particularly useful for

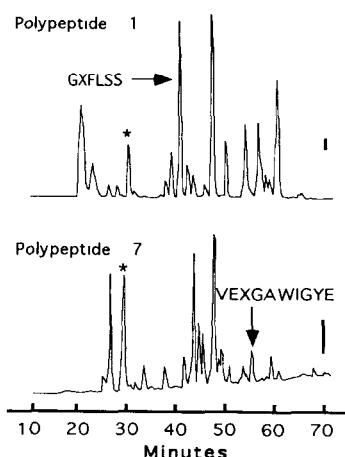


Fig. 3 HPLC separation of tryptic fragments of β -crystallin polypeptides with indistinct NH₂-termini following insolubilization by calpain II. Polypeptide 1 (Fig. 1C) was identified as a partially degraded β B3, with an intact COOH-terminus, based on the sequence of the indicated COOH-terminal tryptic fragment. Polypeptide 7 (Fig. 1C) was derived by partial degradation of either β A3 or β A1, based on the sequence of the indicated internal tryptic fragment. The bar on the right indicates 0.05 absorbance units at 216 nm. X indicates the position of cysteine which was not determined.

Table I

The majority of β -polypeptides insolubilized by calpain II have the same cleavage sites at their NH₂-termini as insoluble β -crystallin polypeptides from cataractous lens

| Polypeptide ^a | NH ₂ -termini ^b | Identity ^c | Found in cataract ^d |
|--------------------------|---------------------------------------|-------------------------------|--------------------------------|
| 1 | Indistinct ^e | β B3 (~ 10) | + |
| 2 | GAPEQA | β B3 (-5) | + |
| 3 | GGLGGS | β B3 (-17) | + |
| 4 | SHGGLG | β B3 (-15) | - |
| 5 | Blocked ^f | β B3 | + |
| 6 | TLPTTK | β A3 (-11) | + |
| 7 | Indistinct ^e | β A3/A1 (-17 to -21) | + |
| 8 | Blocked ^f | β A1 | - |
| 9 | XTKSAG ^g | β A4 (-18) | + |
| 10 | ELPPGS | β B1 (-49) | + |

^aThe numbers correspond to the polypeptides marked in Fig. 1C and D.

^bPolypeptides subjected to direct Edman sequencing from PVDF membranes.

^cPolypeptides were identified by comparison of Edman sequencing results to published sequences of β -crystallin polypeptides [2-4]. In cases where NH₂-termini were blocked or indistinct, the identification was based on the sequence of tryptic fragments. Number in parentheses gives number of residues missing from the NH₂-terminus.

^dBased on similarity of NH₂-terminal sequence and migration during two-dimensional electrophoresis (compare Fig. 1C and D), these polypeptides were also found in the insoluble protein of rat lenses with selenite-induced cataract [9].

^eThese polypeptides contained several NH₂-termini, and were identified by sequencing tryptic fragments (Fig. 3), however, based on Edman sequence data, the approximate cleavage sites at the NH₂-termini are given.

^fThese polypeptides remained blocked, and were identified as intact β B3, and β A1, based on their position during two-dimensional electrophoresis, and similarity of their tryptic maps to the tryptic maps of intact β B3 and β A1 (Fig. 2)

^gX indicates position of cysteine which was not determined.

study of lens proteins, because intact α - and β -crystallins are normally NH₂-terminally blocked, and we wished to identify the intact rat β -crystallins before their breakdown by calpain. While the positions to which many β -crystallins from other species migrate during electrophoresis are known, a direct comparison to identify rat crystallins was not possible. This is because orthologous crystallin subunits from various species can migrate to different positions during two-dimensional electrophoresis. For example, in bovine and human lens, β B1 is the most basic β -crystallin subunit [13,14], while in rat, β B3 is the most basic β -crystallin subunit. Also, without this technique, we could not have identified several partially degraded crystallins which contained indistinct termini following calpain cleavage. This technique will greatly facilitate the study of proteolysis and other post-translational changes in lens crystallins during aging and cataract formation.

The reason β -crystallins lose their solubility following

removal of their NH₂-terminal extensions is unknown. It is also difficult to formulate a hypothesis for calpain induced insolubilization, because the function of the NH₂-terminal extensions of β -crystallin subunits remains unclear. It has been suggested that NH₂-terminal extensions in β -crystallins subunits could interact intermolecularly to stabilize oligomers [15]. While recent data on the structure of β B2 homodimers suggests that NH₂-terminal extensions are not used to stabilize β B2 dimers [16], it still remains plausible that NH₂-terminal extensions in other β -subunits could be responsible for dimer-dimer interactions [17]. If this is true, then insolubilization may result from decreased stability of the β -subunits following their dissociation when the NH₂-terminal extensions are removed. Alternatively, insolubilization following removal of NH₂-terminal extensions may occur because the extensions function as spacers to prevent unwanted interactions between β -crystallin oligomers. A third possibility is that the extensions may be required to maintain the proper secondary structure in individual β -crystallin subunits. Regardless of the mechanism of insolubilization, β B2 subunits appeared less susceptible to insolubilization, since they were more soluble than other β -crystallin subunits following their partial degradation.

Two recent unpublished findings are relevant to the present work. We recently found that physiological concentrations of KCl will delay the precipitation of partially degraded rat β -crystallin subunits by 48 h. This suggested that the initial interaction between partially degraded β -crystallin which causes insolubilization may be ionic in nature. Additionally, incubation of human lens soluble proteins with calpain did not cause significant precipitation of the resulting partially degraded β -crystallin subunits. This result could be due to the lower relative concentration of β B3, and higher concentration of β B2 in human lens, compared to rat lens.

Determining the mechanism of insolubilization is important, because it will not only provide important information regarding the mechanism of cataract formation, it will also assist in determining the function of NH₂-terminal extensions in various β -crystallin subunits. The relevance of partial degradation of β -crystallin subunits in human cataract remains unknown, but is currently under study.

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